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Large-Scale Production and Physicochemical Characterization of Human Immune Interferon

M. P. LANGFORD, J. A. GEORGIADES, G. J. STANTON, F. DIANZANI, AND H. M. JOHNSON*
Department of Microbiology, University of Texas Medical Branch, Galveston, Texas 77550

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Large-scale production of crude high-titered ($10^{2.3}$ to 10^4 U/ml) human immune interferon (type II) was carried out in roller bottle cultures of human peripheral lymphocytes by using the T-cell mitogen staphylococcal enterotoxin A. Over 99% of human immune interferon was destroyed by pH 2 or heat at 56°C for 1 h. The interferon was not neutralized by antibody to human leukocyte interferon. The kinetics of development of the antiviral state were slow for immune interferon relative to those for leukocyte interferon. Ultrogel AcA 54 chromatography of crude or the concentrated interferon resulted in two peaks of activity, a major one (87% of recovered activity) with a molecular weight of 40,000 to 46,000 and a minor peak of molecular weight 65,000 to 70,000. The column elution buffer consisting of 18% ethylene glycol and 1 M NaCl in phosphate-buffered saline resulted in at least 100% recovery of added interferon. The data suggest, then, that the interferon produced under large-scale conditions was immune (type II). The efficiency of the production was comparable to that described for large-scale production of human leukocyte interferon. Our large-scale production system for human immune interferon offers a feasible approach to preparation of large quantities of purified immune interferon for structure studies, antibody production, and clinical application.

The human and mouse interferon systems can be provisionally classified into two groups. These are the virus type (type I) and immune (type II) interferons. Virus type interferons are classically induced by viruses or synthetic polynucleotides (12, 17), whereas immune interferons are usually induced in primed lymphocytes by specific antigen or in unprimed lymphocytes by T-cell mitogens (14, 20, 30, 32, 36). Virus type interferons which are stable at pH 2 are heterogeneous, and at least two antigenically distinct types exist (16, 25). They are called fibroblast and leukocyte interferons, indicating their cellular source. Immune interferon is labile at pH 2 and antigenically distinct from virus type interferon (19, 32). The antigenic relationship of mitogen-induced and antigen-induced interferons is not known.

Considerable success has been obtained in the purification of mouse virus type interferons produced by C-243 cells (8), by Ehrlich ascites tumor cells (21), and by L cells (7, 22, 27). Similar success has been achieved in purification of human leukocyte interferon (1, 18, 29, 34, 35), human lymphoblastoid cell interferon (4), and human fibroblast interferon (1, 10, 18, 23, 34). In some cases, the interferons may have been purified to homogeneity (8, 21, 23, 29).

Of particular interest is the successful large-scale production of human leukocyte interferon (5) for use in clinical trials involving viral infections (15) and neoplasia (26, 33). We describe here a system for large-scale production of human immune interferon by using the T-cell mitogen staphylococcal enterotoxin A (SEA) and present the physicochemical characterization of this interferon. Mouse immune interferon preparations have been reported to possess dramatic antitumor activity in mouse sarcomas (6, 31). Large-scale production of human immune interferon, then, is a prerequisite to physicochemical characterization, purification, and ultimately clinical application.

MATERIALS AND METHODS

SEA. SEA was produced and purified by the Microbial Biochemistry Branch, Division of Microbiology, Food and Drug Administration, Cincinnati, Ohio (2). SEA migrated as a single band on sodium dodecyl sulfate-polyacrylamide electrophoresis at a molecular weight of approximately 28,000 and eluted from a Sephadex G-75 column as a single, symmetrical peak.

Protein determination. Proteins were routinely quantitated by absorbance at 280 nm on a spectrophotometer. On a selective basis protein concentrations were measured by fluorometric assay (3). Fluorescamine was obtained from Roche Diagnostics, and bovine serum albumin obtained from Calbiochem was used as a standard.

Interferon assay. Interferon was assayed in a microtiter system on human WISH cells as described previously (24), except that Sindbis virus (50 to 100 50% tissue culture infective doses per 0.1 ml) was used as challenge virus. Interferon activity was expressed in terms of the National Institutes of Health reference interferon. One unit of interferon is defined as the concentration that resulted in 50% reduction in cytopathogenic effect.

Large-scale production of immune interferon. Blood (usually 100-ml volumes) was collected in acid-citrate-dextrose solution by venipuncture from healthy volunteers. Peripheral blood lymphocytes were isolated by the Ficoll-Hypaque gradient method (24). Lymphocytes were also obtained by plasmapheresis through the facilities of the University of Texas Medical Branch Hospital blood bank on a selective basis. The lymphocytes were suspended to 10^6 cells per ml in RPMI medium. SEA, the immune interferon inducer, was added to a final concentration of 0.1 μg/ml. 2-Mercaptoethanol was added to a final concentration of 10^{-5} M. It improves cell viability, but not interferon yield. Fetal calf serum was added to a final concentration of 10%. Roller bottles (2,000-ml capacity) were seeded with 300 ml of the cell suspension. The bottles were gassed for 1 min with a defined gas mixture (7% O_2, 10% CO_2, 83% N_2). The tightly capped bottles were placed on a roller apparatus and rotated at 8 rpm at 37°C for 4 days. The supernatant was collected after centrifugation at 2,000 rpm in a Sorvall RC-5 centrifuge (GSA head) at 4°C and stored at −70°C.

Concentration of immune interferon. Controlled-Pore Glass beads (CPG-10, Electro-Nucleonics) were added to the interferon supernatant to a final concentration of 5 mg/ml, and the mixture was stirred at 4°C for 3 h. After standing for 30 min, the top two-thirds of the supernatant was discarded, and the beads containing all of the interferon activity were collected by centrifugation at 1,000 rpm for 10 min at 4°C. The beads were washed three times with phosphate-buffered saline (PBS, 0.15 M), pH 7.2, after which the interferon was eluted from the beads with 50% ethylene glycol in 1.4 M NaCl and PBS (J. Georgiades et al., manuscript in preparation). The eluted immune interferon was exhaustively dialyzed against PBS and used in characterization studies.

Removal of SEA inducer from concentrated immune interferon. SEA was specifically removed when required from concentrated preparations of immune interferon by immunoabsorption. The Cowan strain of *Staphylococcus aureus*, which has a high concentration of protein A in its cell wall, was grown in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) under standard conditions at 37°C for 24 h. The cells were harvested by centrifugation at 10,000 rpm (Sorvall RC-5 centrifuge, GSA head) for 10 min, washed five times with PBS, suspended in PBS, heated at 80°C for 5 min, and stored at 4°C in 0.5% Formol-saline. Before use the stored cells were washed three times with PBS and packed by centrifugation. The protein A of the cells specifically bound immunoglobulin G by the Fc portion (13). A 1-ml amount of packed cells was mixed with 1 ml of rabbit hyperimmune antisera to SEA (R. Bennett, Food and Drug Administration), and the mixture was incubated at 4°C for 3 h. The *Staphylococcus*-anti-SEA complex was washed three times with PBS and incubated at 4°C for 24 h with concentrated immune interferon at a 1:10 volume ratio of adsorbent to interferon. The adsorption was monitored by the addition of trace amounts of ^{125}I-labeled SEA to the concentrated interferon preparation. Radioactivity was measured on a Nuclear Chicago model 1185 gamma scintillation counter.

Gel filtration of immune interferon on Ultrogel AcA 54. An Ultrogel (LKB Instruments Inc., Rockville, Md.) column (2.5 by 60 cm) was equilibrated with PBS or other buffers as indicated in Results, and UV absorbance was monitored at 280 nm (ISCO model UA-5 absorbance monitor). The column was standardized with known-molecular-weight substances (blue dextran, bovine-serum albumin, ovalbumin, and myoglobin). Interferon which had been exhaustively dialyzed against PBS at 4°C was loaded on the column and pumped through at a rate of 10 to 15 ml/h. Fractions (5 ml) were collected.

pH and temperature studies. Immune interferon and reference human leukocyte interferon (supplied by the National Institute of Allergy and Infectious Diseases, National Institutes of Health), 3,000 to 6,000 U/ml in RPMI, were reduced to pH 2.0 by 6 N HCl and incubated at room temperature. At various times 0.1-ml volumes were removed and added to 0.9 ml of HEPES buffer (*N*-2-hydroxyethyl piperazine-*N'*-2-ethanesulfonic acid), which raised the final pH to 6.8. The samples were tested for residual interferon activity.

Immune and leukocyte interferons were also tested for heat stability. The interferons in RPMI media were heated at 56°C for various time intervals, after which residual antiviral activity was determined.

Antibody neutralizations. Rabbit antibody to human leukocyte interferon was obtained from National Institute for Allergy and Infectious Diseases, National Institutes of Health. A single dilution, previously determined to be capable of neutralizing 6,000 U of human leukocyte interferon, was incubated with various concentrations of immune interferon for 1 h at room temperature. The samples were then assayed for residual interferon activity.

RESULTS

Production. Interferon yields from individual roller bottle cultures of Ficoll-Hypaque lymphocytes stimulated with SEA varied from $10^{2.3}$ to 10^4 U/ml with a median yield of 10^3 U/ml (Table 1). These maximum yields occurred with 3 to 4 days of incubation as reported previously for SEA (24). By criteria described below such as pH sensitivity, kinetics of antiviral action, and antibody neutralization, all of the detectable interferon produced under the above conditions was immune type (type II). Similar results were obtained with leukocytes obtained by plasmapheresis. Five hundred milliliters of blood, then, with an average of 1.25×10^9 lymphocytes, would be expected to produce approximately

TABLE 1. *Production of human interferon by peripheral lymphocytes stimulated with SEA and phytohemagglutinin-P*[a]

Mitogen	Median concn (U/ml) (range)	Total U from 100 ml of blood[b]	U/10^3 viable cells
SEA[b]	1,000 (200–10,000)	2.5×10^5	1.0
PHA-P	300 (100–600)		0.3

[a] Cultures were stimulated for 4 days with 0.05 μg of SEA per ml and 10 μg of phytohemagglutinin-P (PHA-P) per ml.

[b] Data from 8 donors with 100 ml of blood yielding, on the average, 250 ml of lymphocytes at 10^6/ml in RPMI.

1.25×10^6 U of immune interferon with SEA as the inducer. In previous studies with human peripheral lymphocytes, SEA was found to induce about three times more immune interferon than phytohemagglutinin-P and concanavalin A (24). Under the larger scale roller culture conditions, SEA induced three to five times more interferon than phytohemagglutinin-P (Table 1). Human serum albumin, 1%, could be used in place of fetal calf serum with about 60% of the interferon yield.

pH stability. Immune interferon was tested along with leukocyte interferon for stability at pH 2.0. The results are presented in Fig. 1. As can be seen, immune interferon lost approximately 99% of its antiviral activity in 30 to 60 min, whereas leukocyte interferon was relatively unaffected under the same conditions. Interferon controls incubated under the same conditions, except at pH 7.2, did not show a significant loss in activity. Antibody to human leukocyte interferon did not neutralize the residual immune interferon activity (Table 2). The pH stability data, then, suggest that the SEA-induced interferon is immune interferon (37).

Heat stability. The immune interferon was also tested for heat stability at 56°C along with human leukocyte interferon (Fig. 2). Both interferon preparations lost approximately 99% of their activity in 3 h. The susceptibility of fibroblast interferon to heat is well established (37). The susceptibility of SEA-induced immune interferon to destruction at 56°C is in agreement with data reported for phytohemagglutinin-induced immune interferon (11). There are reports that antigen-induced immune interferon is resistant to heating at 56°C (37). It is possible that mitogen-induced and antigen-induced immune interferons possess different heat stabilities.

Gel filtration chromatography. Immune interferon, 300,000 U (3.3 $\log_{10}$ U/mg of protein) in 5 ml, eluted as two peaks from an AcA 54 Ultrogel column (Fig. 3), corresponding to molecular weights of 40,000 to 46,000 and 65,000 to 70,000. Elution was with 18% ethylene glycol and 1 M NaCl in PBS with 131% recovery of interferon activity. Of the recovered interferon activity, 87% was in the 40,000 to 46,000-dalton peak, whereas 13% was in the 65,000- to 70,000-dalton peak. The 40,000- to 46,000-dalton peak of interferon was purified approximatley 200-fold (5.6 $\log_{10}$ U/mg of protein). When PBS was used as the elution buffer, a similar pattern of elution was observed, except that only 42% of the added

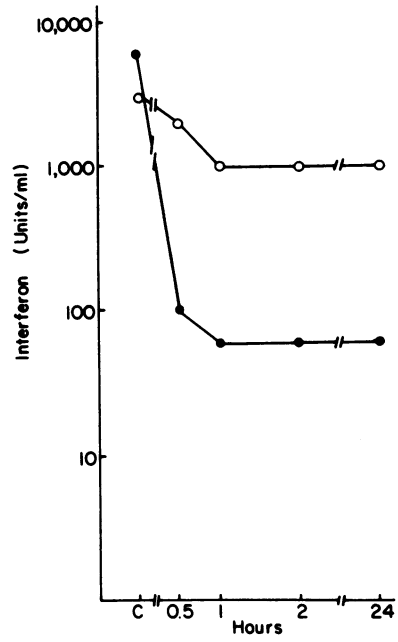

FIG. 1. *Sensitivity of human immune interferon (●) and human leukocyte interferon (○) to pH 2 as a function of time. Controls (C) represent interferon concentrations before lowering of pH.*

TABLE 2. *Neutralization of human interferons with antibody to human leukocyte interferon*

Interferon	Antiviral activity (U/ml)[a]	
	Interferon alone	In presence of anti-leukocyte interferon
Leukocyte	1,000	<10
Immune	100	100
Immune (residual activity after pH 2 treatment)[b]	60	60

[a] Interferon incubated for 1 h at 37°C in culture media or with 600 U of anti-leukocyte interferon before titration for residual antiviral activity. One unit of antibody will neutralize 10 U of human leukocyte interferon.

[b] A total of 6,000 U/ml reduced to 60 U after pH 2 treatment as shown in Fig. 1.

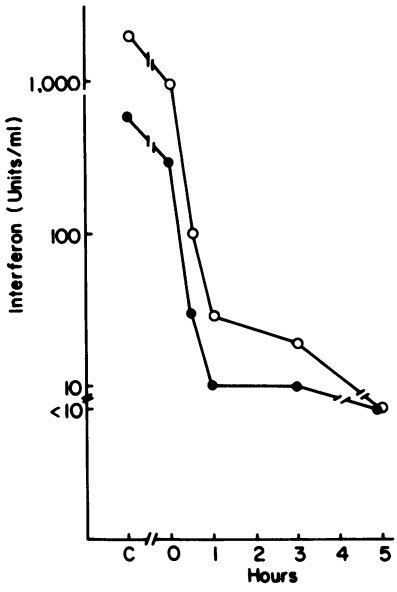

FIG. 2. *Sensitivity of human immune interferon (●) and human leukocyte interferon (○) to a temperature of 56°C as a function of time. Controls (C) represent interferon concentrations before raising of the temperature.*

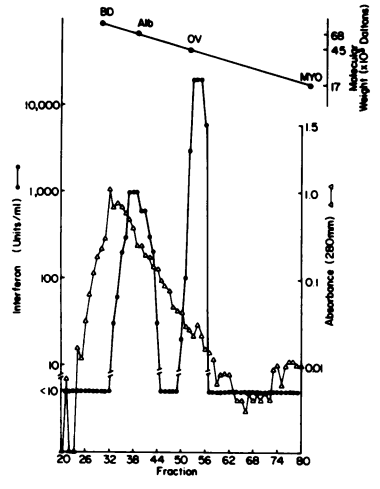

FIG. 3. *Filtration of human immune interferon by Ultrogel AcA 54 column chromatography. The column was loaded with concentrated interferon (300,000 U/5 ml) and washed with a buffer consisting of 18% ethylene glycol and 1 M NaCl in PBS. Fractions (5 ml) were collected. Molecular weight standards were: blue dextran (BD), 300,000; bovine serum albumin (BSA), 67,000; ovalbumin (OV), 45,000; and myoglobin (MYO), 20,000.*

interferon was recovered. The elution buffer of Fig. 3, then, resulted in good recovery.

Kinetics of antiviral activity. It was previously shown that human immune interferon and human leukocyte interferon induced the antiviral state in human diploid cells at different rates, with immune interferon possessing a slower rate (9). Furthermore, the kinetics of leukocyte interferon induction of the antiviral state are not affected by the presence of immune interferon (9). Human immune interferon produced under large-scale conditions and human leukocyte interferon were compared for their relative rates in induction of the antiviral state in human WISH cells (Fig. 4). In agreement with the earlier studies, large-scale-produced human immune interferon at comparable interferon activities induced the antiviral state at significantly slower rates than leukocyte interferon. The relative slowness of the development of protection with even 100 U of human immune interferon suggests the absence of detectable leukocyte interferon in the immune interferon preparations.

Antibody neutralization. The possibility was considered that leukocyte interferon can be induced in human peripheral leukocytes by T-cell mitogens. The SEA-induced interferon, then, was compared with human leukocyte interferon in anti-leukocyte antibody-neutralization tests (Table 2). At the concentrations used, anti-leukocyte interferon completely blocked the antiviral activity of leukocyte interferon, but had no effect on the antiviral activity of SEA-induced immune interferon. Furthermore, the residual antiviral activity of the immune interferon from the pH studies of Fig. 1 was not neutralized by anti-leukocyte interferon. Thus, no evidence of leukocyte interferon in the immune interferon preparations was observed.

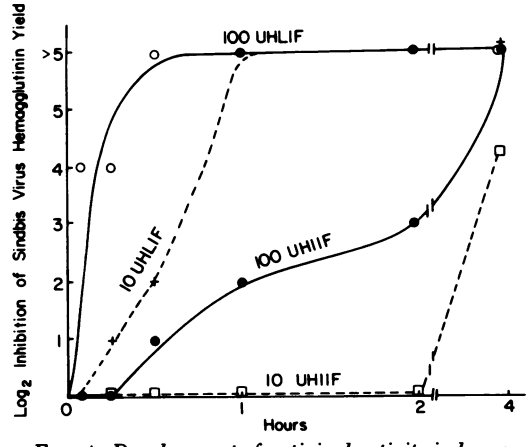

FIG. 4. *Development of antiviral activity in human WISH cells treated for various periods of time with the indicated concentrations (units per milliliter) of human immune interferon (HIIF) and human leukocyte interferon (HLIF). Interferon activity was measured by a decrease in the hemagglutinin yield of Sindbis virus as described elsewhere (9).*

DISCUSSION

We have presented here the first in vitro system for large-scale production of human immune interferon. This, we feel, greatly reduces the problems of purification and the possible use of immune interferon in clinical studies. This system is similar to the previously described mouse system (28), except human peripheral lymphocytes were used instead of mouse spleen cells. We feel that the most important factor for consistently high interferon yields is the use of SEA. Results have been inconsistent, and yields have been three to five times lower for concanavalin A and phytohemagglutinin-P inducers under large-scale culture conditions. 2-Mercaptoethanol (10^{-5} M) results in increased cell viability after a 4-day culture, but is not an essential ingredient for high interferon yield. Restimulation of cells with fresh media and SEA resulted in further production of immune interferon, but at 1/10th the yield of fresh cells (data not shown). The use of cells obtained by Ficoll-Hypaque gradient or by plasmapheresis did not alter the interferon yield. The system, then, is stable and reliable if SEA is used as the inducer.

All of the detectable interferon is immune (type II) in this large-scale system. Treatment at pH 2 resulted in 99% loss of activity in 1 h, whereas the control leukocyte interferon was only slightly affected (Fig. 1). Antibody to human leukocyte interferon had no effect on immune interferon, whereas completely neutralizing the antiviral effect of leukocyte interferon (Table 2). Furthermore, the residual immune interferon activity from pH 2 inactivation was not neutralized by anti-leukocyte interferon antibody. It was previously shown that human leukocyte interferon and human immune interferon produced on a small scale had different kinetics of induction of the antiviral state (9). A comparison of leukocyte interferon with human immune interferon produced in large scale here showed that the immune interferon induced the antiviral state in human WISH cells at the slow rate characteristic of immune interferon (Fig. 4). Furthermore, if as little as 1 U of leukocyte interferon was present in 100 U of immune interferon, it would have been detected by the kinetic curve (data not shown). Taken together, then, the above observations indicate that the large-scale system described here produced high yields of human immune interferon devoid of detectable leukocyte interferon.

Human immune interferon did not bind to hydrophobic columns under conditions that mouse immune interferon and virus-type interferons were bound (data not shown) (28). Thus, differences may exist between human immune and other interferons. More than 99% of both immune and leukocyte interferon was destroyed in 1 h at 56°C. This is in agreement with findings with PHA-induced human immune interferon (11). Antigen-induced mouse immune interferon has been reported to be stable at 56°C (37). This may indicate a difference in immune interferon induced by mitogens as compared with antigen induction. Certainly, heat stability data should be used with caution in classification of these interferons.

Heterogeneity of the human immune interferon was shown by gel filtration chromatography on an AcA 54 Ultrogel column. Two peaks with apparent molecular weights of 40,000 to 46,000 and 65,000 to 70,000 were obtained with 18% ethylene glycol in 1 M NaCl and PBS for elution. About 87% of the interferon activity was in 40,000- to 46,000-molecular-weight range with 200-fold purification. All of the added interferon was recovered with this elution buffer, and this is a routine observation in our gel filtration experiments. PBS elution gave a similar elution pattern, but less than 50% of the added interferon activity was recovered.

In the mouse system ammonium sulfate was found suitable for concentration of immune interferon (28). This is not the case for human immune interferon. Salt precipitation results have been variable and unpredictable. Nonprecipitable antiviral activity has been observed, for example. We found that Controlled-Pore Glass beads were efficient at binding human immune interferon for concentration under bulk culture conditions. A definitive treatment of this system in conjunction with purification of human immune interferon is in the preliminary stage of development (J. Georgiades et al., manuscript in preparation).

Finally, we have presented here a system for production of human immune interferon which is as efficient and functional as that currently used to produce human leukocyte interferon for clinical trials in viral infections and cancers (5). In the mouse system immune interferon is about 100 times more effective as an antitumor agent against mouse osteogenic sarcoma than is fibroblast interferon (6). If a similar relationship were to exist in the human system, then our large-scale production system for human immune interferon could have considerable clinical importance.

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